University of Alberta

Evaluation of nose-only inhalation chamber for aerosol deposition in mouse lungs and comparison of experimental results with mathematical simulation.

by

Venkatareddy Nadithe

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of *Master of Science*

in

Pharmaceutical Sciences

Faculty of *Pharmacy and Pharmaceutical Sciences*

Edmonton, Alberta

Spring 2003

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VenkataReddy Nadithe
Ap# 206
11012 Street, 82 Avenue
Edmonton, T6G 2P6
Alberta, Canada.

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University of Alberta

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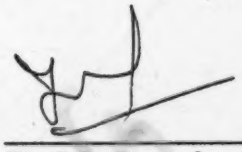
The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Evaluation of nose-only inhalation chamber for aerosol deposition in mouse lungs and comparison of experimental results with mathematical simulation* submitted by *Venkatareddy Nadithe* in partial fulfillment of the requirements for the degree of *Masters of Science* in *Pharmaceutical Sciences.*



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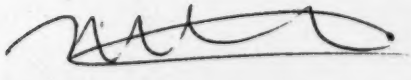

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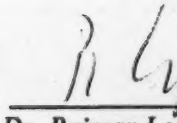

Dr. Raimar Loebenberg

Date: April 9, 2003

ABSTRACT:

In vivo small rodent efficacy testing of new synthetic and biological molecules for the pulmonary route requires an efficient delivery device. For this purpose, a nose-only inhalation chamber was used to deliver aerosolized aqueous compounds to the respiratory tract of the mice. The aim of the study was to determine the efficiency of dose delivery and deposition in the lungs of the mice after using this chamber. A secondary goal was to compare the experimental lung deposition results with the values predicted by mathematical simulation. Experimental tests were conducted by generating aerosols of a radiolabeled formulation of human serum albumin (HSA) with a mass median aerodynamic diameter (MMAD) of 3.9 ± 0.5 μm and a geometric standard deviation (GSD) of 1.43 ± 0.05 by using PARI LC STAR jet nebulizers. Based on the total activity placed in the nebulizer, the chamber delivered 0.108 ± 0.027% to the mice and 0.0087 ± 0.0021% to their lungs. *In vivo* lung deposition was found to be 8.19 ± 3.56% of the total activity deposited in the mouse. Mathematical simulation predictions ranged between 5.89% and 4.40% for various breathing patterns and did not differ significantly from the *in vivo* results ($p > 0.10$). These results provided important quantitative information relevant to aerosol delivery experiments in mouse models. Our results also suggested that the nose-only inhalation chamber would benefit from significant changes to increase the efficiency of deposition in mice so that this chamber can be used for the nebulization of more expensive therapeutic drugs.

ACKNOWLEDGEMENTS

I thank everyone who contributed either directly or indirectly to my research. Particularly, I wish to extend my gratitude to my supervisors Dr. John Samuel and Dr. Warren H. Finlay for providing facilities in their laboratory and directing my project by providing ongoing advice and input. I am also grateful to Dr. John Mercer and John Wilson for their advice in radioactive experiments. Special thanks also go to Dr. Muhib Rahmatalla for helping me with mathematical simulations and Biljana Grgic, Helena Orszanska for helping with techniques in aerosol delivery.

I appreciate all my lab colleagues, for their advice and discussions during both the experimental work and thesis preparation. Finally, I am indebted to all my friends and family members with out whose encouragement this work would not have been possible.

TO MY PARENTS: Sujatha & Srinivasreddy Nadithe

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ABBREVIATIONS

^{99m}Tc	Meta stable Technetium-99
CFC-11	Trichlorofluoromethane
CFC-114	Dichlorotetrafluoromethane
CFC-12	Dichlorodifluoromethane
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disease
DPIs	Dry powder inhalers
FRC	Functional residual capacity
g	Grams
GBq	Giga Becquerels
GIT	Gastro intestinal tract
GSD	Geometric standard deviation
HCl	Hydrochloric acid
HEV	High endothelial venules
HFC-134a	1, 1, 1, 2-tetrafluroethane
hrs	Hours
HSA	Human Serum Albumin
IL-2	Interleukin-2
ITLC-SG	Instant thin-layer chromatography, silica gel
MBq	Mega Becquerel's
MDIs	Metered dose inhalers
mg	Milligrams

min	Minutes
min^{-1}	Per minute
mL	Millilitres
MMAD	Mass median aerodynamic diameter
MMD	Mass median diameter
NOD	Non-obese diabetic
p53	Tumor suppressor gene
PDA	Phase doppler anemometry
SCID	Severe combined immuno deficiency
SD	Standard deviation
Sec	Seconds
$t_{½}$	Half life
µg	Micrograms
µL	Microlitres
µm	Micrometre

CHAPTER 1: INTRODUCTION

1.1 Aerosol delivery and methods for aerosol generation

Drugs are administered to patients by different routes. For over a century aerosol delivery has been an important mode of delivery for targeting lungs, one of the most attractive routes in drug delivery. Aerosol therapy has been widely used in treating diseases such as asthma, pulmonary infections, chronic obstructive pulmonary disease, emphysema, cystic fibrosis, and gene therapy. "Inhalation Aerosols" are defined as colloidal systems consisting of very finely subdivided formulations dispersed in a gas. For lung delivery, the particles can be solids or liquid droplets as long as they are in the respirable range (for droplets, between 1-5 μm). The aerosol droplets between 2-5 μm are deposited in the upper respiratory tract. The particle droplets of less than 2 μm are deposited in the alveolar region, and droplets of less than 0.5 μm are exhaled. Any particles greater than 10 μm are deposited in the throat (Hickey 1996). However, these are not strict cut-off diameters but depend on the flow rate and other deposition factors.

Several techniques are employed for the generation of aerosol droplets. The droplets are generated in sprays by means of atomization, in which the bulk fluid is dispersed first, and then the breakdown of droplets takes place under the influence of physical properties such as surface tension and viscosity. Other methods of droplet formation are by pressure forces due to oscillatory piezoelectric crystals in ultrasonic nebulizers, the pneumatic method in jet nebulizers, spray drying in spray dryers, the electro-hydrodynamic method, the supercritical phenomenon of the fluids, and the dispersion of powder into the air in dry-powder inhalers. These particle generation

techniques have their own advantages and disadvantages in generation of the particles and depend on the physical environment, design characteristics of the device, and formulation-related factors of the drug (Hickey 1996; Finlay 2001).

1.2 Importance of aerosol delivery

Delivering modern drugs based on proteins, peptides (Byron & Patton 1994) and nucleic acids, by the pulmonary route by aerosolization offers many advantages over the conventional routes of administration (Hensel & Lubitz 1997; Agu et al 2001). The lung is an important site for delivering various therapeutic aerosols because of the large surface area available for drug action in the lower respiratory tract. Usually, smaller amounts of a drug, than would be needed otherwise, will suffice to prevent or treat a disease, and the adverse reactions caused by this route of administration are usually less compared to those caused by the systemic route of delivery. Aerosol delivery by this route is also attractive because of the predictability of the drug dose and because this route provides an ideal way to deliver drugs non-invasively for many chemotherapeutic drugs such as IL-2 (Auricchio et al 2002; McCann 2002). The most important therapeutic use of administering drugs by aerosols is rapid absorption (Patton 1997) across the pulmonary mucosal epithelial surface (Figure 1). This rapid absorption helps in achieving a quicker therapeutic effect and results in lower toxicity to other organs. Lung hindrances for drug absorption are few compared to the hindrances in other routes, and the lung offer a friendly environment for most protein and peptide drugs. The permeability of many compounds through the lungs is higher than the permeability through other routes.

The pulmonary delivery permits avoidance of 'first-pass metabolism' associated with the gastrointestinal tract and offers a large surface area for drug absorption (Zheng et al 1999; Butz et al 2002). Drugs given by oral routes may also need more frequent dosing, if the intended site of action is the lungs, and the onset of action will be slow compared to that of drugs delivered to the lungs. Drugs swallowed by the oral route may be digested in stomach enzymes or low pH conditions that pulmonary delivery can avoid (Hickey 1996).

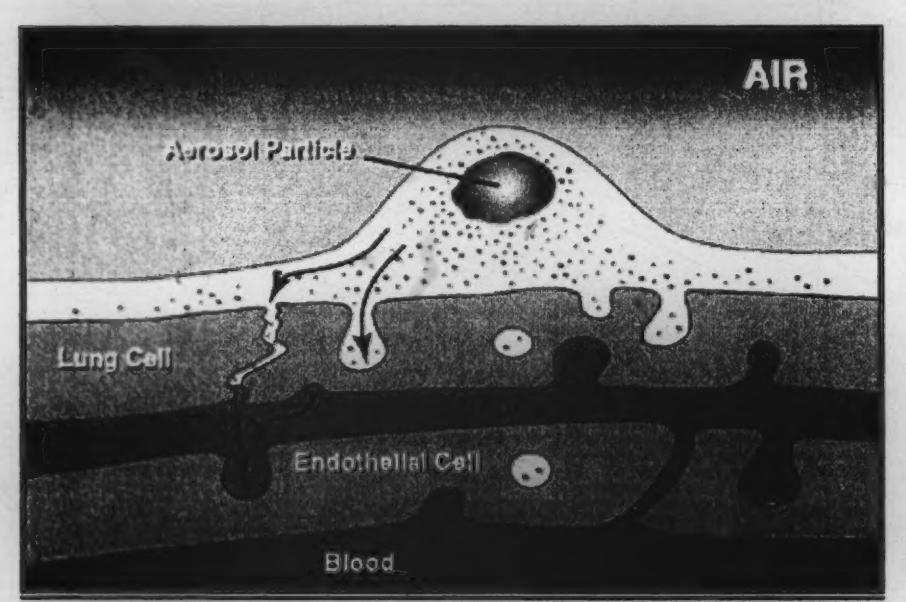

Figure 1: Absorption of macro molecules and their transport into the bloodstream in the deep lung (Patton 1997)*.

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For asthmatic patients, β_2- agonistic drugs given by pulmonary route are more effective due to immediate drug action in the lung. This action results in immediate bronchial dilation and thus alleviate the symptoms (Atabai et al 2002). This route is also used to deliver aminoglycosides (e.g., amikacin) in pulmonary infections, and most studies have shown that the concentration of the aerosolized drug is greater than the minimal inhibitory concentration (Donald et al 2001). The lungs can also be an important route for inducing anesthesia in animals by delivering inhalant anesthetics (Sato et al 2002). In cases of chronic obstructive pulmonary disease (COPD), mucolytics delivered by this route are more efficient than those delivered by other routes (Poole & Black 2000). Aerosol inhalation also offers a number of advantages in gene therapy for lung disease such as cystic fibrosis (Pai & Nahata 2001), in which cystic fibrosis transmembrane conductance regulator (CFTR) is defective, and emphysema, in which α_1- antitrypsin is defective (Allen 1996).

Because of the large number of professional antigen presenting cells such as dendritic cells and macrophages localized in the lung mucosal surface, the pulmonary route may also be advantageous for vaccine delivery (Hensel & Lubitz 1997). The localized pulmonary macrophages can move to different regions of the lung such as pleural, interstitial, alveolar, intravascular, and intraepithelial. These cells constitute a significant percentage of the cells in alveoli and represent the main cellular host defense mechanism in the alveolar space. Macrophages and dendritic cells wander through the lung and are the only alveolar clearance mechanism for particulate material that has escaped the tracheobronchial filters. To leave the lungs, these cells may either migrate to the nearest bronchiole and exit via the mucociliary escalator or may pass into the

interstitium and exit via the blood vessels or lymphatics, often accumulating in the regional lymph nodes (Stockley 1997).

Antigen internalization usually takes place in the periphery i.e., lungs in this case, and process, and present them to lymphocytes for induction of protective immune responses (Nicod et al 2000). Immune responses generated in the lung may also provide protective effects at the mucosal surfaces of other organs because of the cross-communication between the different sites within the common mucosal immune system (Lamblin et al 2000). Lyphyocytes from the lung emigrate to other organs and thus form part of the general route of lymphocyte trafficking. The lymphocytes will also return to the circulating pool either via lymphatics and finally the thoracic duct or directly to the blood (Stockley 1997). This emigration helps to explain why pulmonary administration of vaccines can be used for generating immune responses.

However, in a few instances, the drug deposited in the lungs may not be available for therapeutic action there, possibly because of mucociliary clearance, lung enzymatic activity, or other factors such as the diseased state of the lung. Other situations occur in which the drugs given by the pulmonary route are absorbed quickly, so that frequent dosing of large doses is required. Local irritation or toxicity may also become an issue when using some types of drugs. Nevertheless, if the drug must be delivered by only the pulmonary route, the circumstances can be changed by using an appropriate delivery system and made suitable for pulmonary administration. Encapsulating the drugs in liposomes and delivery by the pulmonary route will produce pulmonary controlled

release (Khanna et al 1997), thus avoiding the need for frequent dosing. In some cases where the drug is not suspended or stable in the solution for aerosolization, it can be encapsulated and used for nebulization. Various drugs can be targeted to alveolar macrophages and epithelial cells by encapsulating them in liposomes, a process which helps in cellular drug delivery to the lungs. Water insoluble drugs such as 9-nitrocamptothecin encapsulated in liposomes and delivered by nebulization were shown to be effective in cases of human cancer xenografts (Knight et al 1999) showing the importance of lung delivery. Gene delivery vector formulations can also be optimized for pulmonary aerosol delivery if encapsulated or complexed (Densmore et al 1999; Zou et al 2000) in a suitable drug delivery system that provides protection during the aerosolization process.

Thus, the advantages of lung delivery are greater than the disadvantages, except in situations where drugs administered by this route are relatively less potent than other non-invasive or systemically administered drugs (e.g., Theophylline) (Hickey 1996).

1.3 Basic anatomy and physiology of the lung

The lungs are two irregular shaped organs present in the thoracic cavity. The trachea and the two main bronchi indirectly connect them with each other. Gaseous exchange in the alveoli is the primary role of the lungs (Berne 2000). Oxygenated blood leaves the lungs via the pulmonary veins and is pumped by the left ventricle through the systemic arteries to the capillaries, where it is supplied to the tissues (Berne 2000). The lung starts from the trachea and is divided into the conducting and respiratory zones, terminating with smaller airways called "alveolar sacs". Collateral channels present in the

respiratory zone will help to provide an alternate pathways for the airflow if any obstruction is present in the respiratory zone (Hickey 1996).

The conducting zone is devoid of alveolar sacs, and the respiratory zone is devoid of cilia. As the airways divide into smaller divisions, the diameter, the length and the airway caliber decrease, but the surface area or the cross-sectional area increases (Wiebel 1963). The total cross-sectional area of the alveolar surface is 140-160 m^2 in the adult human lungs. C-shaped cartilaginous rings present in the trachea and bronchi will provide résistance to the bulk flow of the air in the lung. Bronchioles are devoid of these structures, so the lower respiratory tract is less rigid and easily collapsible.

Through out the tracheobronchial tree the lungs are covered by thin varied epithelial cells (Figure 2) that provide protection against inhaled particles. In order for drugs to be quickly absorbed without hindrance, the drugs have to be targeted to the lower respiratory tract because of its thinner epithelial cells and lack of ciliated and mucous cells. The mucocilary clearance by the upbeating of the cilia and the mucous secretion in the upper airways provides a protective mechanism and removes unwanted particles from the inhaled air. The epithelial layer has a pH of 6.9 and helps absorb acidic drugs faster than the basic drugs (Hickey 1996). The Type-1 cells present in the alveolar region help in gaseous diffusion, and Type-2 cells produce surfactant. The other types of abundantly available cells are alveolar macrophages, dendritic cells, and mast cells.

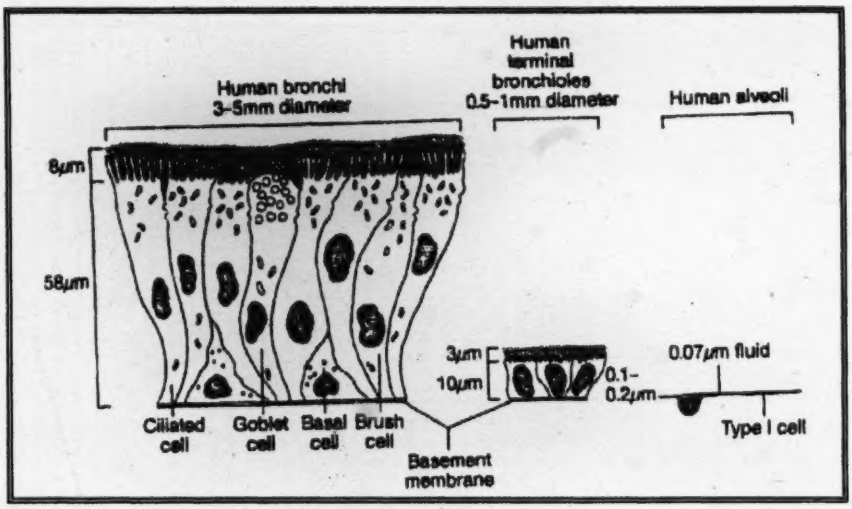

Figure 2: Lateral view of epithelial cell size and surface fluid thickness in different regions of the human lung (Patton 1996)*.

Aerosol deposition depends on the patient's inhalation pattern such as normal or forced breathing. A change in the pattern of breathing will change the volumes of air moving into and out of the lungs. The transportation of drugs in the lungs takes place by different processes such as passive diffusion, facilitated diffusion, and active transport (Adjei 1997). Drug molecules move across the alveolar epithelial cells by transcytosis, pacellular movement, and receptor mediated tanscytosis (Patton 1996), where they are eventually deposited in the underlying interstitial space. The molecules may then enter the lung lymphatic pathways, or may be absorbed into local capillaries, through permeation via the capillary membrane, a process that provides systemic effects (Saltzman 2001). The macromolecules' absorption into the blood has been found to be

* Reprinted from *ADVANCED DRUG DELIVERY REVIEWS, Vol 19, 1996, pp 3-16, Patton JS: "Mechanisms of macromolecule absorption by the lungs"*, with permission from Elsevier.

inversely related to the molecular weight of the deposited molecules in the lungs (Hastings et al 1992).

1.4 Importance of mouse models in aerosol therapy

Laboratory mice have been used extensively as human surrogates in health research for studying the therapeutic efficacy and toxicity of drugs. Because of their small size, short generation time, and low cost of maintenance, many mice can be used in controlled experiments within a brief period of time. Mice have been genetically and immunologically more extensively characterized than any other laboratory animal. In many ways, the murine immune system resembles that of humans. As well, the availability of several in-bred strains of mice has been a major contributing factor in the recent advances in immunology and vaccine research (Goldsby et al 2000). Immunodeficient strains such as the severe combined immuno deficiency (SCID) mouse permit the reconstitution of the human immune system in mice by administering human immune cells to them (Berney et al 2001). The Transgenic and Knockout mouse models have been developed to serve as models for human diseases (Brodie & Deng 2001; Davidson & Rolfe 2001; Hock & Lamb 2001). In some instances, a particular transgenic mouse may be the only animal model suitable for developing novel therapies against a particular disease. For example, the MUC1-transgenic mouse model is the only small-animal model available for investigating therapeutic cancer vaccines designed to overcome self-tolerance against human MUC1 (Rowse et al 1998; Samuel et al 1998).

However, the pulmonary delivery of aerosol to the mice is technically challenging because of their small tidal volumes. Delivering aerosol to mouse lungs without its deposition in the nose and gastrointestinal tract is difficult. In contrast to humans, the

commonly used laboratory animals such as rats, mice, hamsters, and guinea pigs are all obligate nose breathers. Because of the close apposition of their epiglottis to the soft palate, they cannot inhale through the mouth. As well, differences in lung anatomy and physiology might lead to differences in aerosol particle deposition between mice and humans (Rao & Verkman 2000). The position of the lung in humans is vertical where as the lungs are horizontal in mice during inhalation. The smaller tidal volumes of mice result in lower aerosol deposition rates than those in humans (Table 1). The deposition of aerosols in mice depends on the aerosol particle size, which is affected by the delivery device, formulation parameters, and the breathing pattern (McCallion et al 1996; Finlay 2001). For the tidal breathings, the optimal particle size for alveolar deposition is 3 μm in both humans and mice, but for tracheo-bronchial deposition, it is approximately 6 μm in humans and 4 μm in mice (Stahlhofen et al 1989; Hsieh et al 1999).

Table 1: Respiratory parameters in humans and mice, adapted from (Rao & Verkman 2000).

Animal	Minute ventilation or flow rates (mL/min)	Tidal volume (mL/breath)	Respiratory or breath frequency (breaths/min)
Humans	6000	500	12
Mice	1.06 – 1.108	0.007 – 0.01	106 -163

Despite the above technical difficulties, mice are used as models for investigations involving the aerosol delivery of therapeutic agents in the treatment of pulmonary diseases. For example, the aerosol delivery of genes to the lungs was shown to result in the expression of the transgene in mouse pulmonary epithelial cells, demonstrating the potential of this approach for gene therapy for cystic fibrosis (Gautam et al 2000). Delivery of insulin to the lungs has the advantage of delivering the drug to the systemic circulation without enzyme degradation. A diabetic mice model, non-obese diabetic (NOD), is used for studying the aerosol delivery of insulin for insulin-dependent diabetes mellitus (Harrison et al 1996). For studying the aerosol delivery of corticosteroids for treatment of asthma, an asthmatic mouse model is an appropriate choice (Chapman et al 1998). A murine metastatic lung carcinoma model was used to demonstrate the anticancer effects of the aerosol delivery of tumour suppressor gene p53 along with liposomal 9-campothecin delivered to the lung as an aerosol (Gautam et al 2002). The number of experiments involving aerosol delivery in mice models of human diseases is expected to increase continuously.

1.5 Aerosol delivery devices

The inhalation system currently available generate aerosols in the range of 0.5 to 5 μm, with their exact size depending on their deposit site, protect the physical and chemical stability of the drug; and allow for the reproducibility of drug doses during aerosolization. Three different types of commercially available aerosol delivery devices exist for generating pharmaceutical aerosols: nebulizers, metered dose inhalers, and dry powder inhalers. The amount of delivery that can be achieved by using any of these

devices is between 15-40% of the emitted dose (Anderson 2001). These pharmaceutical aerosols delivered to the lungs will produce mainly localized and systemic effects.

1.5.1 Metered dose inhalers (MDIs)

MDIs were most popular in the late 1950's. These aerosol devices have a container, a metering valve, and the liquefied propellants with any excipients, and the drug in solution or suspension, with an actuator providing a mouthpiece for inhalation. Upon actuation, the drug in the propellant is expelled from the pressurized canister by the metering valve. The liquefied propellant creates the energy required to aerosolize the drug into rapidly evaporating droplets. The surfactants used in the formulation will help in the suspension of the drug particles in these devices. The drug can be dissolved in the propellant by using surfactants and co-solvents such as ethanol. The common propellants (chloroflurocarbons) used in these devices are trichlorofluoromethane (CFC-11) dichlorodifluoromethane (CFC-12), and dichlorotetrafluoromethane (CFC-114), which can be combined in different ratios to get the required vapour pressure, liquid density, and solvency. These propellant systems also have less pulmonary toxicity, high chemical stability, purity, and compatibility. Because of environmental concerns, these propellants have been replaced with hydrofluroalkanes such as 1, 1, 1, 2-tetrafluroethane (HFC-134a) (Langley et al 2002). Proper coordination between the actuation and inhalation, the design of the valves, increasing the physical stability of the drug in the propellant, optimal particle exit velocity, and the reproducibility of the doses from these devices will have a considerable effect on the efficient deposition of aerosols in the lungs (Hickey 1996; Finlay 2001).

1.5.2 Dry powder inhalers (DPIs)

DPIs are the devices that can aerosolize the dry powder particles. More than one mechanism is involved in the generation of aerosols from the dry-powder inhalers. The basic features of the DPI are a dose-metering mechanism, an aerosolization mechanism, a deaggregation mechanism, and an adaptor to direct the aerosol to the patient's mouth. The powdered drug particles for this application have to be less than 5 μm in aerodynamic diameter. Proper milling is required before a drug is aerosolized. However, these small particles are difficult to disperse because of the electrostatic forces, Vander Waals, and capillary forces that are present in them. Often, these drug particles are dispersed with an excipient such as lactose, which is larger than the drug particles and acts as a carrier. During the aerosol generation process, these particles are stripped off from the carrier and cannot cross beyond the mouthpiece or throat (Hickey 1996).

The patient's inspiratory effect and inspiratory duration will influence the aerosol generation and deposition. The other important factor in the delivery of a drug from these devices is the powders' characteristics such as size, shape, moisture content, chemical composition, and surface charge. Dose counters in some of the inhalers will help the patient to decide whether the drug has been delivered or not. Thus, the efficient delivery of powders by using these systems depends on the powder's physiochemical characteristics, device design, and the patient's breathing pattern (Hickey 1996; Finlay 2001).

1.5.3 Nebulization

Nebulizers are used mainly to deliver solutions and suspensions in large volumes. These devices are simple to use and without the difficulties associated with MDIs and DPIs. With a MDI, coordination between breathing and aerosolization is important, whereas this kind of coordination is not essential in nebulization. The different types of nebulization are distinguished from each other by the source of the particle generation. Jet nebulizers and ultrasonic nebulizers are the most common types of nebulizers, which are available commercially in large numbers.

1.5.3a Jet Nebulization

In a jet nebulizer (Figure 3), the compressed gas produces aerosol droplets. A jet nebulizer has a liquid reservoir where the solution or the suspension is lodged, and compressed dry or wet gas flows from the cylinder and passes through the venturi of the nebulizer. This activity causes the fluid from the nebulizer to rise along the walls of the feed tube. At the end of the feed tube where the gas exits, the Bernoulli effect creates a negative pressure because of the air's rapid expansion, which results in the rise of the liquid as thin ligaments into the feed tube and collapse into primary droplets of aerosol. Some droplets move out from the nebulizer, and the remainder returns to the reservoir fluid containing the non-respirable bigger droplets, which hit baffles before their return into the reservoir. The primary aerosol leaving the nebulizer may be significantly changed by the aggregation or solvent evaporation and condensation of the aerosol droplets (O'Callaghan & Barry 1997).

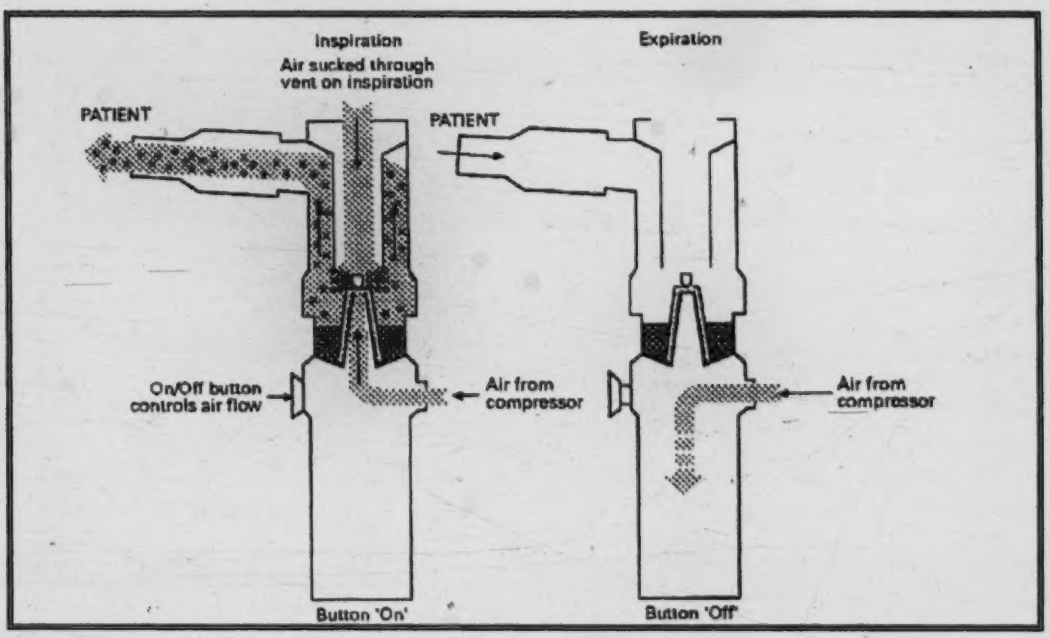

Figure 3: A closed jet nebulizer (e.g., Pari LC STAR). It produces aerosol only when the patient presses a button allowing compressed air to pass through the nebulizer (O'Callaghan & Barry 1997)*.

Jet nebulizers are of two types based on the closing and opening of the vent. The advantage of the open-vent nebulizer (Figure 4) is the extra amount of air that enters the nebulizer during inspiration. This air helps remove more of the small droplets generated quickly for inspiration than the closed vent nebulizer does and also reduces wastage of aerosol during exhalation, but these results depend on the patient's aspiratory flow. In the closed-vent nebulizer, the same amount of aerosol is generated but takes longer to nebulize (McCallion et al 1996; O'Callaghan & Barry 1997).

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* Reprinted from *Thorax, 52 (suppl 2), 1997, S31-S44, O'Callaghan C, Barry PW: "The science of nebulised drug delivery"*, with permission from BMJ Publishing Group.

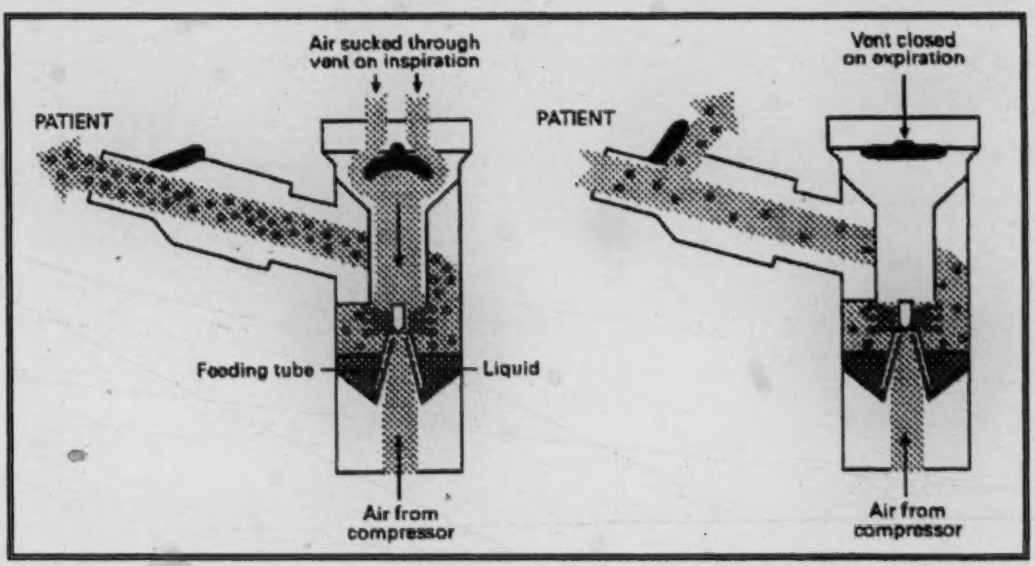

Figure 4: An open-vent jet nebulizer (Pari LC jet plus). Nebulization times for this device are faster, and the drug dose received by the patient is significantly greater than those of the conventional nebulizers, but the times are not as fast as those of the open nebulizer (O'Callaghan & Barry 1997)*.

1.5.3b Ultrasonic Nebulization:

Ultrasonic nebulizers (Figure 5) use ultrasonic energy for the production of droplets during nebulization. A rapidly vibrating piezo-electric crystal at high frequency produces ultrasonic waves. These energy waves are transferred from the bottom of the solution or suspension in the nebulizer to the surface of the formulation. When a sufficient amount of energy has been acquired, the formation of droplets takes place. Like jet nebulizers, ultrasonic nebulizers have baffles that aid in the removal of the larger droplets from the aerosol droplets (Zierenberg 1992; Taylor & McCallion 1997).

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Two theories offer explanations for the droplet formation. The capillary wave theory states that the excited liquid on the surface results in the formation of capillary waves, which under sufficient energy, break down into therapeutic droplets for the inhalation. The cavitation theory states that the droplets are produced by hydraulic shocks produced by the cavitation bubbles. The droplet formation results from both the capillary waves' help in the formation of the droplets, and the cavitation bubbles' help in their initiation and subsequent production (Taylor 1998). Different nebulizers have different aerosol output rates because of the variations in the design of the nebulizers. The particle size and stability of the formulations are changed during nebulization.

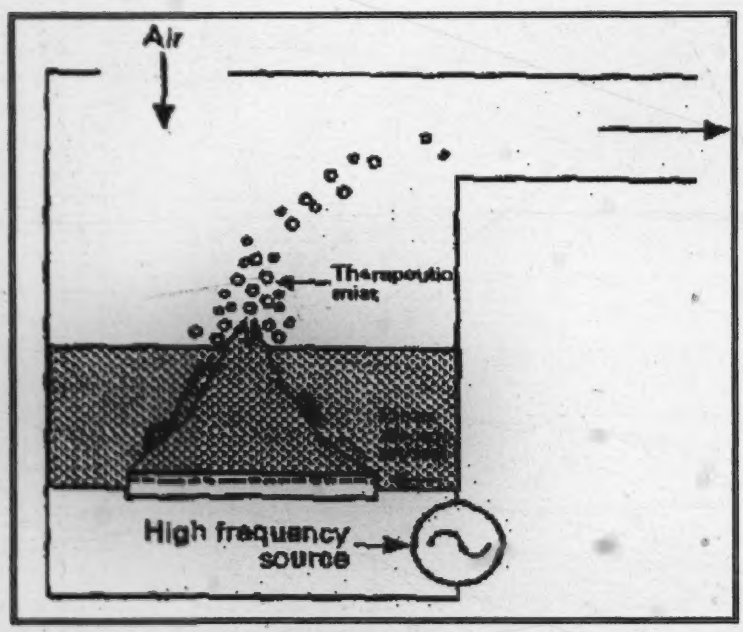

Figure 5: Schematic diagram of an ultrasonic nebulizer, showing vibration of fluid (O'Callaghan & Barry 1997)*.

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1.6 Choice of aerosol generation device

Aerosol delivery devices should provide an adequate drug dose to the lungs, be simple to operate, minimize oropharyngeal deposition, and match the *in vivo* requirements (Dhand 2000). The choice of an aerosol delivery system depends on numerous factors such as the drug and the characteristics of the aerosol generator, including many of the experimental factors. If the drug is present in solution, the drug can be delivered by either metered dose inhalers or nebulizers. If long, continuous aerosolization is needed for delivering large volumes or high doses of a drug, nebulization is the only available choice (Hess 1995; Cipolla et al 2000). If the drug has to be delivered in powder form, then dry-powder inhalers are the choice. An ultrasonic nebulizer should not be used to aerosolize a drug suspension sensitive to ultrasonic waves. A pilot scale experiment is usually necessary to choose the suitable aerosol device for a particular drug of choice. The compatibility of the drug with the delivery device should also be ensured before choosing an aerosol delivery device (Anderson 2000). Finally, large differences in output characteristics exist among nebulizers (Geller 1997). Thus, the challenge is to develop suitable devices in terms of efficacy, safety, and convenience for the next generation of inhalers (Lotvall 2001).

However, all theses delivery devices have limitations, which may affect their clinical efficacy. These limitations include the dependency of the lung deposition of dry powders on the inspiratory flow rate and the need for the reformulation of MDIs with CFC-free gases (Roche & Huchon 2000). Therefore, the choice of an aerosol delivery device depends on the choice of the drug to be delivered, along with many other factors.

1.7 Pulmonary drug delivery methods for mice

The different pulmonary methods or devices available for *in vivo* pulmonary drug delivery studies using a mouse model are instillation, insufflators, and inhalation chambers.

1.7.1 Instillation

Instillation is a primitive and relatively easy method of administering a drug to the lungs. With this method, the mouse is anaesthetized, and a few drops of the drug are instilled into the mouse's nostrils. The amounts of the drug that reach the mouse's lower respiratory tract will less than those of other methods because of the physiological barriers in the nose and upper respiratory tract. As well, this method cannot achieve a uniform and deep penetration of the drug throughout the lungs.

1.7.2 PennCentury insufflators

Recently, a new device has become available for aerosolizing solutions, suspensions, and powdered drugs into the trachea of rodents. It design shows an acutuating syringe, side arm, sample chamber and a deliver tube suitable for delivery of powdered drug into the trachea of rats (Banker 2002). A similar device is also available for aerosolizing solutions or suspensions in mice. The use of these devices for immunological studies must be considered with utmost care because insufflating the aerosol into all the mice in a group is technically difficult. As well, determining whether the drug has been instilled into trachea or the gut is difficult unless the tracheal region is opened for visual inspection. Doing so may not be possible in all the experimental set-ups because an additional surgical procedure is required. Sometimes, the mice can be

harmed if the trachea is destroyed and inflammation results. Moreover, the amount of the insufflated drug can vary among the mice in a group. The above complications are prominent when the PennCentury insufflators are used on mice.

1.7.3 Inhalation chambers

Inhalation exposure chambers are widely used in the inhalation toxicology of rodents. These chambers permit easy breathing and also accurate simulation of normal conditions, but are expensive to construct and maintain. In whole-body exposure chambers, aerosol is delivered into a box where mice are allowed to inhale with their entire bodies immersed in the aerosol cloud (Peebles et al 2001). This method is simple, does not require anaesthetization or restraining of the animal, and large number of animals can be exposed to aerosols all at once. The disadvantage of these systems is the large deposition of the aerosolized drug all over the animal's skin. This deposition may lead to subsequent ingestion by mouth and unwanted or toxic responses. Animals may tend to avoid aerosol exposure in these chambers by huddling together or by covering their noses with their own fur. As well, significant loss of aerosol particles in these chambers can result from the particles' impaction on the walls of the chambers (Phalen et al 1984).

1.7.4 Nose only inhalation chambers

Alternatively, nose-only inhalation chambers were developed to avoid the above problems with small animals like rats and mice (Boogaard et al 2000; Ramesh et al 2001). Currently, these chambers are the only suitable devices available for conducting experiments involving a large number of mice and requiring simultaneous exposure of

the aerosol. Nose-only inhalation chambers can be designed according to the number of mice needed for a single exposure and for anywhere between 6 and 100 or more mice. The validation of these nose-only inhalation chambers is an important step towards their use in inhalation studies (Pauluhn 1994; Bonnet et al 2000). The primary advantage of nose-only inhalation chambers over conventional inhalation chambers is the avoidance of drug deposition all over the mouse's skin surface during the aerosolization process. Unfortunately, little data is available in the literature on the fraction of the drug that deposits in the mice and their lungs when such chambers are used.

1.8 Factors affecting aerosol deposition in the lungs

Capillaries represent the most extended endothelial target surface area in the lungs, making capillaries an important and privileged target for site-specific delivery of therapeutic agents. The lungs' vascular surface area is larger than that in other organs, so that drug absorption can be greatly increased. Approximately 30% of the total endothelium in the body belongs to the pulmonary vasculature. The factors governing aerosol deposition, which may lead to absorption, have to be carefully considered because they will ultimately affect the therapeutic effect of the drug deposited.

1.8.1 Mechanism of particle deposition

The aerosol particle is influenced by mechanical and electrical forces after it has been generated and is traveling in the respiratory tract. However, pharmaceutical particles are usually not heavily charged, and mechanical forces dominate the deposition mechanism. The three important mechanical mechanisms by which the aerosol is deposited into the lungs of animals are impaction, sedimentation and diffusion (Lieutier-Colas 2001; Washington 2001). All three mechanisms act on the particle simultaneously,

but impaction and sedimentation are important for the deposition of larger particles. An aerosol particle continually changes its velocity and direction while it is traveling to deep inside the lungs.

Inertial impaction is the primary mode of deposition for particles greater than 1μm diameter and is predominant in the upper respiratory tract where the aerosol particles are traveling with high velocity and have high density. A particle with a high mass, diameter, and velocity is more likely than other particles to the walls of the lung surface in the upper respiratory tract because of the law of inertia. Generally, inertial impaction increases with particle size, density and flow.

Sedimentation plays an important role in the lower respiratory tract deposition of the aerosol particles. The particles sized greater than 1 μm will settle on the lung surface under the influence of gravitational force. This method of deposition will increase as the duration of the breathing cycle increases. Every collision of a particle with a gaseous molecule changes the kinetic energy and direction of the motion (displaced) and, as a result, the particle moves in a random direction through the gas. This kind of the movement is called as "Brownian motion" or "diffusion", which increases with the duration of the breathing cycle. This type of deposition occurs in the peripheral alveolar region where the air flow is low or absent (Finlay 2001; Hillery et al 2001)

1.8.2 Physiological factors

Physiological factors such as tidal volume, breath holding, type of breathing, inspiratory flow rate, and lung morphology, and diseased state (Yeh et al 1976; Chamberlain et al 1983; Katz et al 2001) play a pivotal role in the deposition of aerosol particles in the lungs of animals. With an increase in tidal volumes, more air enters the

lungs, and thus, more aerosols are carried into the deep lungs, resulting in higher tracheo-bronchial and alveolar deposition. During the normal inhalation pattern, tidal volumes are constant in a mouse, but they can change in conditions like stress. The type of breathing also affects the amount of drug deposited in the lungs of an animal. In contrast to what occurs in mouth-breathing animals, most of the drug is deposited in the nose and pharynx of nose-breathing animals. Lung morphology shows that the airways decrease in diameter and length as they approach the periphery, so that the particle path in the terminal airways is short before the particle is deposited. Each bifurcation results in more chances for the particle to be deposited by impaction. In diseased states such as asthma, where a bronchial obstruction exists, the chances of the particle being deposited in the lower airway decreases. In general, lung regions with poor ventilation because of air flow obstruction or low compliance will receive a lower volume of a delivered aerosol than other regions. Coordination of the aerosol generation device with the breathing pattern increases the pulmonary deposition by facilitating sedimentation and diffusion, breath holding will aid in optimal pulmonary drug delivery because the particle has a chance to settle on the surface of the lungs.

One of the most important physiological factors influencing aerosol particle deposition is the inspiratory flow rate (Zhang et al 2000). Increasing the inspiratory flow rate will increase the turbulence and result in deposition by impaction. This impact is higher in the larynx and trachea and decreases in the bronchi and alveoli. Faster inspiratory flows increase oropharyngeal and upper airway deposition by inertial impaction; slower inspiratory flows minimize oropharyngeal and upper airway deposition and enhance distal delivery and deposition through sedimentation and diffusion.

However, fast inspiratory flows may be necessary with some dry-powder systems to deaggregate and disperse the dry powder. Thus, an optimal inspiratory flow rate will help to achieve maximum aerosol deposition in lungs. Other factors include particle velocity, inspiration volume and inspiration time during inspiration (Hillery et al 2001; Banker 2002)

1.8.3 Formulation factors

The particle size of aerosol is conventionally expressed as the particle's aerodynamic diameter, which is the diameter of the spherical particle with a unit density that settles at the same rate as the described particle. This is the square root of the density of the particle multiplied by particle diameter. The smaller the particle size, the higher the deposition. The mass median aerodynamic diameter (MMAD) gives the median distribution of the aerosol particles by dividing the aerosol mass size into two halves. The geometric standard deviation (GSD) is the size ratio at 84.2% on the cumulative frequency curve to the median diameter that assumes log-normal particle distribution. Particles with a GSD equal to 1 are called "monodisperse aerosols" and those greater than 1 are called "polydisperse aerosols". In practice GSDs less than 1.22 are considered to be "monodisperse". MMAD and GSD are important aerosol parameters that determine the aerosol deposition (DeHaan & Finlay 2001). Aerosols with a higher MMAD deposit in the upper respiratory tract because of their higher momentum. The GSD becomes an important factor because aerosol particles with the same MMAD can have different GSD values. For example, mono-disperse and poly disperse aerosol particles can have the same MMAD but different depositions because these particles have different GSD numbers.

Aerosol velocity is the velocity at which aerosol enters into the lungs. Higher velocities will result in deposition in the upper respiratory tract, and optimal velocities will result in deposition in the broncheo-alveloar region. With metered dose inhalers, the aerosol will be deposited with a higher velocity that is usually greater than the inspirtaory flow rate and will result in higher deposition in the oro-pharynx. Usually nebulizers and dry powder inhalers have some kind of entrainment device, which will reduce the aerosol velocity, which in turn, depends on the inspiratory flow rate and lung physiology of the animal. Most particles have densities around 1 gr/cc. If the particles have a density less than 1 gr/cc, the aerosol will have a smaller aerodynamic diameter than the mean physical diameter. The physical stability of the aerosol is also an important parameter affecting aerosol deposition in the lungs. After the aerosol is generated, it has to be stable before it is deposited in the lungs, but stability may not occur in real situations where inter-particle reactions can take place. With hygroscopic drugs, the aerosol may grow in size in the humid lung environment, resulting in advance deposition. On the other hand, the particle size may decrease because of solvent evaporation resulting in farther deposition the lungs. This result explains why the *in vitro* data obtained at room temperature for particle deposition should be compared with great care before correlating them with the data obtained from the *in vivo* depositions (Finlay 2001; Hillery et al 2001; Washington 2001).

1.9 Mathematical models involving rodents

Mathematical models and numerical predictions may be helpful in the optimal design of animal experiments and in reducing the cost and time involved. In practice, it is important to note that delivery and deposition, while related, are not necessarily linked.

For example, slower regional flows may decrease delivery efficiency but actually enhance deposition through sedimentation and diffusion. In these cases complex computer models are needed to account for these multiple effects in predicting regional aerosol deposition. Because the factors affecting deposition are so complex and difficult to predict, pre-clinical studies of a new drug of interest should be validated by mathematical models that can aid in designing efficient pulmonary drug delivery.

Theoretical predictions for aerosol deposition in the respiratory tracts of rats have been reported recently (Anjilvel & Asgharian 1995) and have been based on by Yeh et al concept of a deterministic lung model (Yeh et al 1979). Phalen performed measurements on casts of mouse lungs and generated data on their morphology (Phalen 1991). The underlying concept in this research was similar to that in the work of Yeh et al (Yeh et al 1979), where the dimensions and number of the alveolar airways were obtained by a numerical interpolation from data for the tracheobronchial region and the most distal alveolar generation. Phalen (Phalen 1991) also presented predictions of deposition probability in the extrathoracic and tracheobronchial regions for various ventilation values and provided comparisons with the mouse-head deposition efficiency of radioactive monodisperse particles as determined by Raabe et al (Raabe 1988). Hsieh et al (Hsieh et al 1999) produced theoretical calculations to predict regional deposition in mice, based on the anatomical lung model of Phalen (Phalen 1991) compared the results with those obtained from rats. In our present work, we developed a mathematical model for predicting the aerosol lung deposition in mice.

A version of this chapter has been published. Nadithe et al.2003. Journal of Pharmaceutical Sciences. 92: 1066-1076.

CHAPTER 2: HYPOTHESIS, AIMS AND OBJECTIVES

2.1 Hypothesis

We hypothesize that nose-only inhalation chamber will help in efficient aerosol delivery to the lower respiratory tract in a mouse model. This will further help in optimizing the pulmonary dose delivery for studying other *in vivo* experiments in the mouse model.

We also postulate that comparing the experimental results with mathematical model will help in validating the newly developed mathematical model for predicting the lung deposition in a mouse model. This will help in predicting the deposition of many drugs delivered by aerosols, without the need for *in vivo* experiments.

2.2 Aims and Specific objectives

The aim of this thesis was to validate the nose-only inhalation chamber for aerosolization experiments. This work was done by using Technetium-99m (99mTc), a short-lived radionuclide used to label a model protein, human serum albumin (HSA), which has a high molecular weight (65 Kilo Daltons) and a long biological half-life, which prevents a too rapid clearance across the lung epithelial cells (Folkesson et al 1996; Nilsson et al 1997). A new computer simulation code was also developed to predict the regional particle deposition in the respiratory tract of mice. This simulation was

compared with the *in vivo* data obtained from aerosol delivery of a radiolabelled model protein. And the specific objectives are:

1) To evaluate the nose-only inhalation chamber for aerosol delivery in a mouse model.

   a) To label a model protein (HSA) for detecting the drug deposition in the lungs by using radioactive material.

   b) To find the *in vitro* stability of the labelled model protein during the aerosolization process.

   c) To find the percentage of drug deposition in mouse lungs immediately after aerosolization.

   d) To find the efficiency of the nose-only inhalation chamber in terms of the amount of drug delivered.

2) To compare the *in vivo* aerosol deposition data with a mathematical model to predict drug deposition in a mouse model.

   a) To find the validity of the data for the experimentally found lung depositions in mice by theoretically predicting the amount by using a mathematical model developed specifically for mouse-lung deposition.

CHAPTER 3: MATERIALS AND METHODS

3.1 Preparation of ^{99m}Tc labelled HSA

Human Serum Albumin (Sigma, St. Louis, MO, USA) was labelled with ^{99m}Tc by a direct labelling technique. To obtain good labelling, the pH of the solution was maintained between 2 and 3 (Zolle et al 1973; De Ligny et al 1976). Briefly, 5 mg of HSA and 60 µg of stannous chloride were dissolved in 2 mL and 100 µL of 0.9% sodium chloride, respectively, and were mixed together and shaken for 30 min. The pH of the resulting solution was brought to 2.35 by using 1 M HCl. The HSA solution was mixed with the required amount (0.545 GBq) of radioactive sodium pertechnetate (Edmonton Radiopharmaceutical Centre, Edmonton, Canada). The so-called "second elution" of sodium pertechnetate was always used in these preparations. The second elution material has a higher specific activity of ^{99m}Tc and a lower concentration of oxidation products from radiolysis. The final solution was kept to a minimum volume and left to incubate for 5-7 hrs.

Free ^{99m}Tc was separated from ^{99m}Tc labelled HSA by using gel permeation chromatography (Dekker et al 1982). Sephadex G 25 columns were used for separation (PD-10 Columns, Supelco, PA, USA). The columns were pre-equilibrated with 0.9% normal saline, and the reaction mixture was allowed to run through the column. Approximately 20 fractions of the prepared sample were collected in different vials. Bound ^{99m}Tc elutes first (fractions 3-7), followed by free technetium. The initial purified sample was diluted up to 15 mL for further nebulization studies.

3.2 Labelling efficiency

The labelling efficiency of the preparation was determined by using thin-layer paper chromatography. A volume of 10-20 μL of samples was placed on instant thin-layer chromatography silica gel (ITLC-SG) paper strips impregnated with silica gel (Gelman Sciences Inc. Ann Arbor, MI) slightly above the 0.9% sodium chloride solvent front. The free technetium moved to the top of the strip, and the labelled protein remained at the bottom. The sample solution was allowed to run for more than 3 min before it was divided into two equal halves for measuring the activity by using a Gamma Counter (Minaxi Auto-Gamma®, 5000 Series; Packard, Frankfurt). The labelling efficiency is defined as

$$\text{Labelling efficiency} = (^{99m}\text{Tc-HSA} / {}^{99m}\text{Tc-HSA} + \text{free } {}^{99m}\text{Tc}) * 100$$

3.3 Decay correction

Decay correction was done by using the equation

$$A = A_o * e^{-\lambda t} \quad \text{or} \quad A = A_o * e^{-(0.693 * t / t_{1/2})},$$

where A is the radioactivity at time't'; A_o is the radio activity at time zero; lambda (λ) is decay constant; t is time and $t_{½}$ is half life of 99m-technetium (6.01 hrs). All the radioactive samples were corrected for the decay correction before they were used in interpreting or analyzing the results.

3.4 Nose-only inhalation chamber and experimental set-up

The inhalation chamber is similar to a commercially available chamber (In-Tox Products, Albuquerque, NM) with 12 plexiglass compartments of 47 mL each for lodging the mice during inhalation. These compartments were designed so that only the noses of the mice were exposed to the aerosol cloud (Figure 6). The upstream side of the

inhalation chamber was connected to a jet nebulizer (Pari LC STAR, Pari, Starnberg, Germany) that produced aerosols by using a compressed air source (DeVilbiss Pulmo-Aide, 5650D, American Allergy Supply, TX, USA). The downstream side was connected to a vacuum pump, which was used to maintain continuous aerosol flow in the system. Aerosol continuity in the chamber was set up by using a flow meter. The pressure in the chamber was maintained at atmospheric pressure by adjusting valves and with constant monitoring by the pressure manometer connected to the chamber (Figure 7).

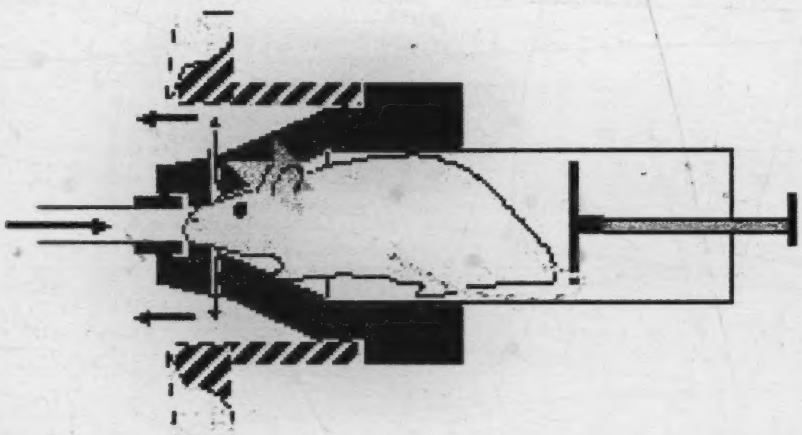

Figure 6: Diagram showing how the mice will inhale in a nose only inhalation chamber (Thorne 2000)*.

* Reprinted from *TOXICOLOGY, Vol 152, 2000, pp 13-23, Thorne PS: "Inhalation toxicology models of endotoxin- and bioaerosol-induced inflammation"*, with permission from Elsevier.

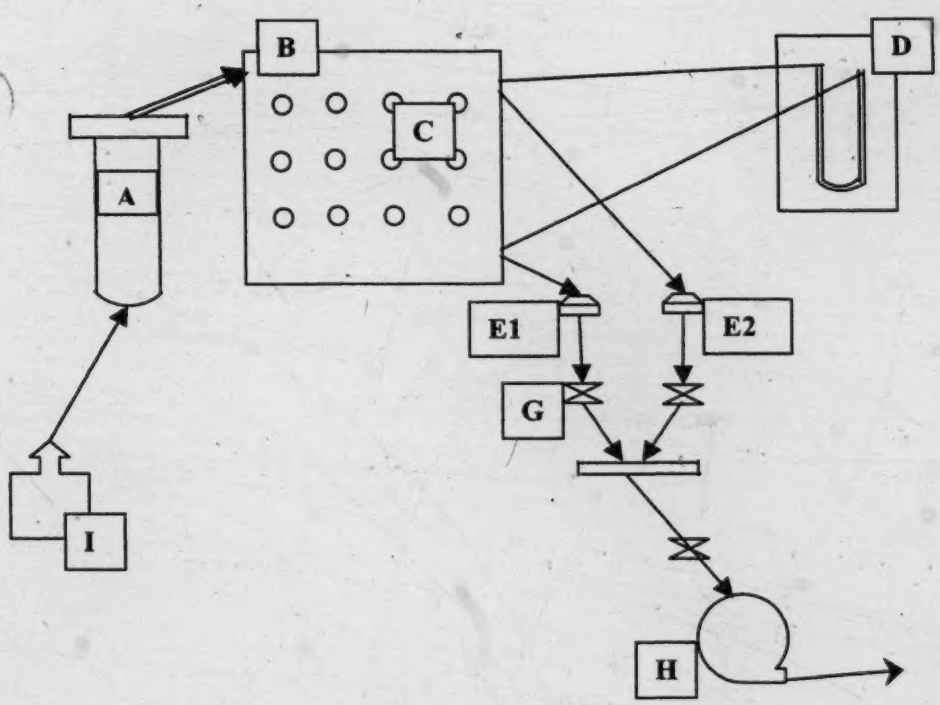

Figure 7: Schematic representation of nose-only inhalation chamber set-up for an *in vivo* test. (A) Nebulizer (B) Inhalation chamber (C) plexy glass chamber for mice (D) Manometer (E1 & E2) Filters (G) Control valves (H) Vacuum pump (I) Compressed air source*.

* Reprinted from *Journal of Pharmaceutical Sciences, Vol 92, 2003, pp 1066 -1076, Nadithe V, Rahamatalla M, Finlay WH, Mercer JR, and Samuel J: "Evaluation of nose-only aerosol inhalation chamber and comparison of experimental results with mathematical simulation of aerosol deposition in mouse lungs"*, with permission from John Wiley & Sons.

3.5 Nebulization

Aerosols were generated by nebulization at room temperature with a PARI LC STAR jet nebulizer using dry compressed air at a flow rate of 4.5 litres/minute. The vent/valve of the nebulizer was closed with Parafilm® to prevent additional air coming into the system. Three millilitres of a radiolabelled HSA sample (1 mg, 41 MBq) were placed into the nebulizer and nebulized for 10 min. For stability testing, the aerosol was collected on three different absolute filters (Respirgard, Marquest Medical Products, Inc., Englewood, USA) arrayed between the nebulizer and the inhalation chamber. The aerosols collected on these filters were washed separately with 5 mL of 0.9% NaCl for further analysis of formulation stability. The filters were weighed before and after nebulization to determine the mass change and to ensure that the nebulization was taking place. *In vivo* deposition studies were performed three times with similar experimental conditions except that 12 C57BL/6 mice with different weights (mean weight 24.01 ± 3.18g) were placed in the plexiglass chambers. After running the nebulizer for 10 min, all the animals were removed from the chamber quickly; then they were immediately euthanized, and their blood and other tissues including heart, lungs, kidneys, head, stomach, and intestines were collected.

3.6 Determination of specific gravity of the aerosols

The specific gravity of the solution was determined by using a refractometer (T/S refractometer, Reichert). A drop of the solution was placed on the slide of the refractometer, and the corresponding specific gravity of the sample was read on the scale.

3.7 Aerosol characteristics

The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the aerosols were determined by using an Aerosizer (Amherst Process Instruments, Inc. Massachusetts, USA) connected to the nose-only chamber opening.

3.8 Calculation of MMAD from aerosizer output data

More than 1, 00,000 aerosol particles pass through the aerosizer during every second. The data for a single run was colleted for 60 seconds. The aerosizer averaged the data for 60 seconds and gave a mean MMD (mass median diameter). These data were used to calculate the particle size (MMAD) of the aerosol.

$$MMAD = \sqrt{(\text{Density of the solution} * (MMD)^2)} \ .$$

3.9 Respiratory tract morphology

The mouse-lung mathematical model dimensions are shown in Table 2. This model consists of 26 generations. The trachea is generation 3, and the alveolar region begins at generation 18. In this table, the Phalen lung model (Phalen 1991) was used and scaled to a functional residual capacity (FRC) of 0.84 mL according to Heish et al. (Hsieh et al 1999). In this model, the extra volume formed by the alveoli was added only to the cumulative volume of the mouse lungs and not to increase the size of the alveolar duct.

The data for the extrathoracic region were obtained from the data of Schreider et al. (Schreider & Raabe 1981), where 35 airway sections of a 330-gram rat were presented from the nares to the end of the larynx. The volume and dimensions of this region were split into two parts, one from the nares to the beginning of larynx and the other from larynx to the trachea. A trapezoidal numerical integration scheme was used to calculate the volume of each part, with each airway section assumed to have a cylindrical shape. The resulting volumes were 0.28 mL and 0.0495 mL, respectively. The total volume of this region was scaled down to a value of 0.0217 mL for the mice.

3.10 Breathing parameters

The various respiratory parameters include tidal volume, breath frequency, and inspiration flow rate. Based on values in the literature (Guyton 1947; Enhorning et al 1998; Hsieh et al 1999), the breath frequency was chosen to vary between 160 min^{-1} to 300 min^{-1}, and the tidal volume was chosen to vary from 0.15-0.289 mL. These values are commonly used by different workers in this field and represent values from actual plethysmography tests for relaxed and distressed mice, respectively.

The following equation determines inhalation flow rate:

Inspiration flow rate (mL/sec) =

Breath frequency (min^{-1}) * Tidal Volume (mL) / 30.0

Table 2: Phalen lung model of a mouse scaled to FRC = 0.84mL, adapted from (Phalen 1991).

Generation	Number of airways	Diameter (cm)	Length (cm)	Bifurcation angle	Gravitational angle	Cumulative volume(mL)
1	1	0.11174	1.794154	0	0	0.017594
2	1	0.110887	0.42735	0	0	0.021721
3	1	0.103337	0.676596	0	0	0.027386
4	2	0.106355	0.383178	14	12	0.03421
5	4	0.085989	0.152366	18	5	0.037729
6	8	0.067886	0.062606	30	13	0.039531
7	16	0.058834	0.073166	12	19	0.042707
8	32	0.054309	0.037714	10	19	0.045496
9	50	0.04752	0.032434	11	30	0.048372
10	75	0.040732	0.032434	10	30	0.051547
11	115	0.033943	0.030172	16	30	0.05468
12	175	0.030172	0.025646	11	30	0.057899
13	270	0.027154	0.024892	12	30	0.061804
14	420	0.021874	0.020366	18	30	0.065023
15	640	0.01584	0.018103	22	30	0.067297
16	980	0.01056	0.013577	33	30	0.068456
17	1505	0.007543	0.009806	61	30	0.0691
18	3010	0.006789	0.008297	45	30	0.070859
19	6020	0.006789	0.007543	45	30	0.074636
20	12040	0.006034	0.007543	45	30	0.081116
21	24080	0.006034	0.007543	45	30	0.091458
22	48160	0.006034	0.007543	45	30	0.122014
23	96320	0.006034	0.007543	45	30	0.17583
24	192640	0.006034	0.006789	45	30	0.280157
25	126858	0.006034	0.006789	45	30	0.440402
26	12500000	0.003771	0.003017	45	30	0.861721

3.11 Deposition models

In this work, the mouse lung is modeled as a series of monopodial bifurcations, where the probability of a particle being deposited in an airway segment depends on three deposition mechanisms: impaction, settling and diffusion. A mathematical model was chosen for each of these mechanisms, and the probabilities were combined to give a deposition probability for a given particle in a given generation of the lung model. For particle impaction, an equation given by Chan and Lippmann (Chan & Lippmann 1980), based on deposition in multi-generation airway casts, and including the effect of secondary flows, was chosen to serve this purpose.

Sedimentation was dealt with by using an equation given by Pich (Pich 1972) and modified by Wang (Wang 1975) to account for airway inclination. The inclination angle for each airway segment in each lung generation was considered when calculating the fraction of particle depositing by settling.

Diffusion is an important deposition mechanism for particles smaller than approximately 1 μm, and an equation based on Ingham (Ingham 1975) was used to estimate the deposition in the airways. The relation for particle deposition in the extrathoracic region was obtained from a fitted equation based on the radiolabelled data of Raabe et al. (Raabe 1988), with correction for the particle clearance occurring during and after deposition (Hsieh et al 1999). This equation is as follows:

$$\text{Probability} = \exp\left(-0.0177 - 0.346 / d_{ae}^{1.5}\right),$$

where d_{ae} is the aerodynamic diameter of the aerosol.

3.12 Input parameters for mathematical model

Some of the input parameters needed for simulating the model are given below.

1) Input type: 1 = nebulizer; 2 = specified aerosol.

2) Aerosol Type: In this experiment the generated aerosol is assumed to be non-hygroscopic aerosol. The basis for our assumption is that when the aerosol is generated we have a large number of droplets per cubic centimeter of aerosol. Thus this will not change the size of the aerosol and assumed to be non-hygroscopic (Finlay 1998; Stapleton & Finlay 1998).

3) Aerosol Characteristics (if input = 2 only in the above parameter)

- MMAD of the aerosol
- GSD of the aerosol
- Approximate number of aerosols per cc in the aerosol
- Bin width (min = 0.1 max = 9.9)

4) Room conditions

- Temperature
- Relative humidity

5) Solution Information

- Initial volume of the solution in the nebulizer.
- Surface tension

6) Breathing information

- Tidal volume
- Inhalation flow rate
- Type of breathing

- Breathing cycle

7) Nebulizer flow rate

8) Lung geometry.

3.13 Statistical methods

To test the significance of the differences between the computer-simulated data and the experimentally determined data for aerosol deposition in the mouse lungs, the Wilcoxon Signed-Ranks Test was used. A Student t-test was used to compare the difference in activity and mass of aerosol delivered. The analyses were performed by using SYSTAT statistical software (version 5.2.1, SPSS Science, Chicago, IL). Significance at a p-value of 0.05 was used.

A version of this chapter has been published. Nadithe et al.2003. Journal of Pharmaceutical Sciences. 92: 1066-1076.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Labelling efficiency and stability

Technetium-labelled HSA was tested for its stability immediately after purification, after dilution of the purified sample and 5 hrs after purification. A five hrs period was chosen because it was long enough to carry out all the deposition studies of the mice from the point where the formulation was purified to the point where the activity in the mouse organs was counted using a scintillation counter. The data are shown in Table 3, which shows the stability was greater than 99%. This result demonstrated that the *in vitro* labelling by the direct labelling method with technetium produced a product that was stable during the 5 hrs period of the experimental studies.

Complexation of technetium to HSA will help in detection of protein in the lungs even if not much protein has been deposited. The use of high molecular proteins in lung-deposition studies will reduce any errors that may be caused by the immediate passage of the small molecular weight proteins across the lungs after deposition. The stability of this complex is an important factor in deposition studies because the free technetium in the sample clears faster from the lung after deposition than the protein labelled with technetium.

Table 3: Mean labelling purity of 99mTc labelled HSA samples at different time intervals after purification (n = 9, ± SD).

Samples at different time intervals	Radiochemical purity* (%)
Purified sample	99.34 ± 0.20
Purified diluted Sample (30 min)	99.40 ± 0.28
Five hrs after Purification	99.44 ± 0.10

* Radiochemical purity indicates the percentage of the total detected radioactivity that is in the form of ^{99m}Tc bound to HSA*.

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4.2 *In vitro* stability of aerosol to nebulization

The stability of the aerosol formulation during nebulization is also important in studying aerosol deposition. The complex formed by the compound and the labelling agent should be stable to the mechanical forces during nebulization (Diot et al 2001). When Technetium-99m binds to HSA, the Technetium-99m will be present in one or all of its stable reduced states such as Tc(III), Tc (IV), Tc (V). This reduced state depends on the pH and type of the reducing agent used, along with many other factors. In studies of aerosols, researchers express contrasting opinions about the *in vitro* stability of the radio-labelled formulations. Waldman et al. found that a higher chemical breakdown of the radio-labelled complex occurs during ultrasonic nebulization than during jet nebulization (Waldman et al 1987) but Groth et al. found, by using a different radiolabelled formulations, that the complex is stable even to ultrasonic nebulization (Groth et al 1989). To study this effect by using our formulation, the aerosols collected from the filter washings were analyzed. The mass of collected aerosol on all the filters is shown in Table 4, which shows that most of the aerosol mass was deposited on filter 1 and 2 and that a negligible amount was present on filter 3. A mass balance existed between the total amount kept in the nebulizer and the total amount deposited on the filters. Table 4 also indicates that all the aerosol mass was deposited on the filters and didn't pass beyond filter 3 and that all the mass was taken into account for *in vitro* calculations.

The corresponding percentage depositions are also shown in Table 4, which shows that 42 % of the mass was nebulized during the nebulization within 10 minutes of nebulization, and that 62% was still left unnebulized. The total aerosolized mass was deposited on filter one, and negligible amounts were deposited on filters 2 and 3. The

percentage activity of the nebulized aerosol solution shown in Table 5 indicates that almost 50% of the solution was successfully nebulized within 10 minutes and that the other 50% was left in the nebulizer. Most of the aerosol particles were deposited on filter 1, whereas only a small fraction and almost nothing were deposited on filters 2 and 3, respectively.

The mass distribution of the nebulized HSA from Table 4 and the measured filter radioactivity from Table 5 also show that the radiolabel accurately measured the aerosol mass. This comparison reveals that both the mass and activity were accounted for in the calculations. No significant difference occurred between the recovered percent mass and recovered percent activity shown in Tables 4 and 5.

Table 4: Mass of aerosol solution present at different regions in *in vitro* setup after nebulization (n = 9, ± SD)*.

Samples	Mass of aerosol solution in grams	Percentage
Initial 3 mL sample in nebulizer	2.97 ± 0.21	100 ± 7.1
Sample left at end of 10 min nebulization	1.79 ± 0.12	60.3 ± 4.0
Filter 1	1.19 ± 0.13	40.0 ± 4.4
Filter 2	0.06 ± 0.05	2.0 ± 1.7
Filter 3	0.01 ± 0.01	0.3 ± 0.3

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Table 5: Percentage of aerosol radioactivity present at different regions in the in vitro setup after nebulization (n = 3, ± SD)*.

Samples	Percentage of total radioactivity
Initial 3 mL sample in nebulizer	100%
Sample left at end of 10 min nebulization	51.03 ± 8.59%
Filter 1	44.21 ± 4.97
Filter 2	5.89 ± 3.09
Filter 3	0.02 ± 0.01

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The labelling stability of the ^{99m}Tc-HSA, as determined by the filter washings after the nebulization, is shown in Figure 8. HSA-bound activity was greater than 97.48 ± 1.61% of the total activity on filter 1, on which the majority of the mass and the activity was deposited. Filter 2 contained 5.89 ± 3.09% of the total activity, of which about 20% represented unbound ^{99m}Tc.

A majority of mass and activity was present on filter 1, and a minority of mass and activity was present on filter 2. The smaller mass on filter 2 represented 20% of the unbound activity on filter 2. This 20% was not the amount of unbound activity that was delivered, for the majority of the aerosolized material trapped on filter 1 had a very low percent of the total unbound activity. The aerosol that was generated and trapped represented a total of 42% of the mass (filters 1 and 2), 49% of the activity, and of the total activity in the samples on both the filters, the bound activity was about 97%. The 20% unbound technetium represents the activity occurring on filter 2 and not to the total unbound activity of the aerosol. This unbound activity made up 3% of the total activity.

The sample left in the nebulizer still contained the bound technetium, indicating that the formulation withstood the jet nebulization. One study has shown that the flow rate can affect the stability of the formulation during the nebulization (Waldman et al 1987). For this reason, any radiolabelled formulation used for aerosol studies has to be optimized for maximum stability to withstand the nebulization so that the formulation does not change during and after nebulization.

These results showed that the formulation of the HSA and the technetium complex was relatively stable and not significantly altered by nebulization. The present study, with our experimental set up at a flow rate of 4.5L/min and using a PARI LC star nebulizer, showed that the stability was greater than 97%, thus indicating that stability can depend on the formulation and the type of the nebulizer used for aerosolization. These *in vitro* results demonstrated that the formulation was suitable for deposition studies because of the negligible amounts of free technetium in the formulation.

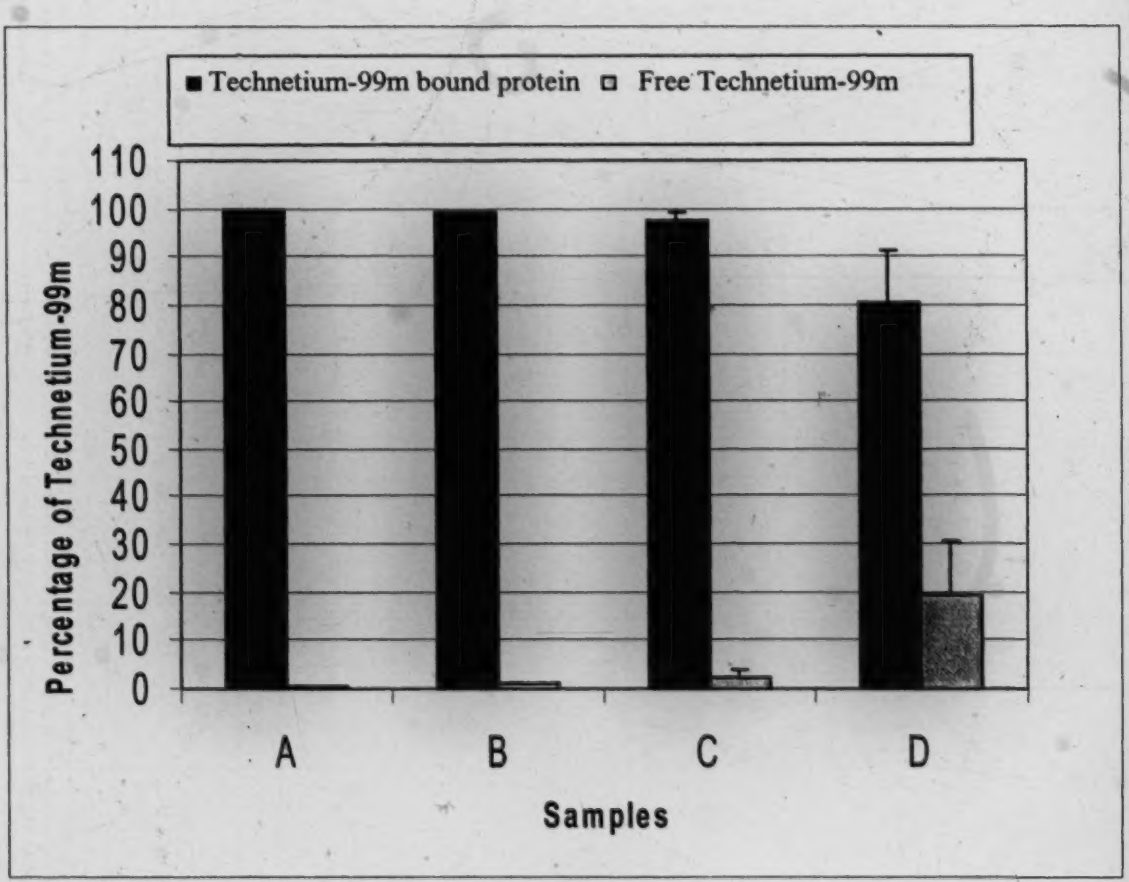

Figure 8: Stability of 99mTc labelled HSA for various samples during nebulization. (A) Initial purified samples (B) Samples left in nebulizer (C) Sample collected on filter 1 (D) Sample collected on filter 2. (n = 9, Error bars indicate ± SD)*.

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4.3 Density of aerosol solution

Density is one of the factors that determine the deposition by effecting the droplet formation and aerodynamic particle size of the aerosol. As well, density also effects the nebulization output from the nebulization. A specific gravity of 1.005 was found, indicating the formulation was slightly denser than water.

4.4 Characteristics of aerosols

The MMAD and GSD calculated from the measured (mass median aerodynamic diameter) MMD and density of the aerosol solution were found to be 3.90 ± 0.5 μm and 1.43 ± 0.05, respectively (mean ± SD; n = 11). These aerosol particle characteristics were obtained from the collected aerosols from the different nose positions, where mice inhale, of the nose-only inhalation chamber. The experiment was performed with the aerosol delivered to the Aerosizer such that the generated aerosol couldnot mix with the ambient (atmospheric) air prior to entering the Aerosizer. The aerosol generated from the nebulizer first enters the main exposure chamber and then travels to the nose-only plexiglass chambers. We sampled the aerosol directly at the location where mice inhale the aerosol (with no dilution from ambient air), thus giving us the particle size of the aerosol actually inhaled by the mice. Particle-size changes within the Aerosizer because of exposure to the ambient air (in the sheath) were negligible, since our independent measurements of the nebulized saline solutions (mesaurements taken by using phase doppler anemometry (PDA), so that no exposure to ambient air occurred during the measurement process) gave the same particle sizes as the Aerosizer measurements.

4.5 Deposition of nebulized aerosol in mice lungs

The *in vivo* deposition data for the mice following 10 minutes of breathing the nebulized ^{99m}Tc-HSA formulation are shown in Figure 9. The vast majority of the nebulized drug was deposited in the head, gastrointestinal tract, and lungs. The high amount of activity in the gastrointestinal tract may have resulted from the initial deposition of aerosols in the nose and throat and that were subsequently swallowed. The head also had a high amount of activity, indicating that is high extrathoracic deposition. As the mice do not inhale through their mouths, the deposition in the extrathoracic region and gastrointestinal tract must have come through the nose. As well, no passive deposition of aerosol was found in the mouths of the mice because the inhalation chamber is designed so that only the nose of the mouse is exposed to the aerosol cloud. Thus, the deposition in our experiments occurred only through the noses of the mice and not by their mouths, either by active or passive means.

Free technetium present in the lungs is known to enter systemic circulation through the permeable lung epithelium. Based on this fact, the amount of the technetium-labelled drug deposited in the mucosal surfaces such as those of the lungs and the gastrointestinal tract should appear first in the heart and then in the blood and the kidneys. Our data showed that of the total activity deposited in the mice's bodies, less than 0.81 ± 0.12 percent was present in the blood and 0.10 ± 0.10 was present in the kidneys. Negligible amounts of activity were seen in other organs such as the heart, spleen, and liver. The very low activity in the blood and the organs of excretion (kidneys and liver) demonstrates that no major clearance from the lungs into the blood stream or

drug absorption from the upper GI tract occurred within the experimental period (10 min).

The aerosolized HSA shows a small loss of label. Recovered material from the filters shows that less than 3% of free technetium is present in the total activity recovered. This amount of free Tc is not expected to significantly alter the deposition characteristics. The short time between deposition in the mice and their sacrifice also precludes significant biological breakdown of the radiolabelled complex. In our radioactive deposition studies with mice there was also negligible activity detected in blood and kidneys further indicating that the activity is retained in the lungs after deposition.

Lung deposition was 8.19 ± 3.56% of the total amount of aerosol deposited in the mice. This result shows that a 40-50% variation occurred in the data. These variations were not caused by the individual positions of the mice in the chamber, for we counted the number of aerosol particles exiting from each position where the mice inhaled the aerosols. The data (954215 ± 71731) showed (Table 6) that no significant difference occurred in the number of particles at different places in the nose-only inhalation chamber. The large intersubject variability in the experimental data may be accounted for by differences in breathing patterns and the respiratory geometry of the individual mice.

The observed depositions in the lungs may be considered as sufficient when individual local action of drug is required or when lung bioavailability cannot be achieved by administering the drug by other routes of administration. However, the deposition pattern shown in Figure 9 may be a concern in certain situations (e.g.,

pulmonary vaccine delivery) because of the high amount of drug deposited in the gastrointestinal tract. This situation might elicit immune responses by both the lung-associated lymphoid tissues and gut associated lymphoid tissues. This result might make determining which route gave the response difficult, unless controlled studies were done by delivering the same amount of dose by the oral route.

Table 6: Number of aerosol particles exiting from each nose-only position in the nose-only inhalation chamber.

Chamber Positions	Number of particles exiting	Particles sampling time (Seconds)
1st	912494	70.01
5th	959483	67.62
7th	968885	67.41
12th	954436	69.8
8th	963011	68.67
11th	1121390	71.28
4th	1028321	68.57
3rd	923334	68.33
2nd	890155	67.51
6th	918852	60.12
10th	856004	70.48
Average particles	954215 ± 71731.07	68.16 ± 2.95

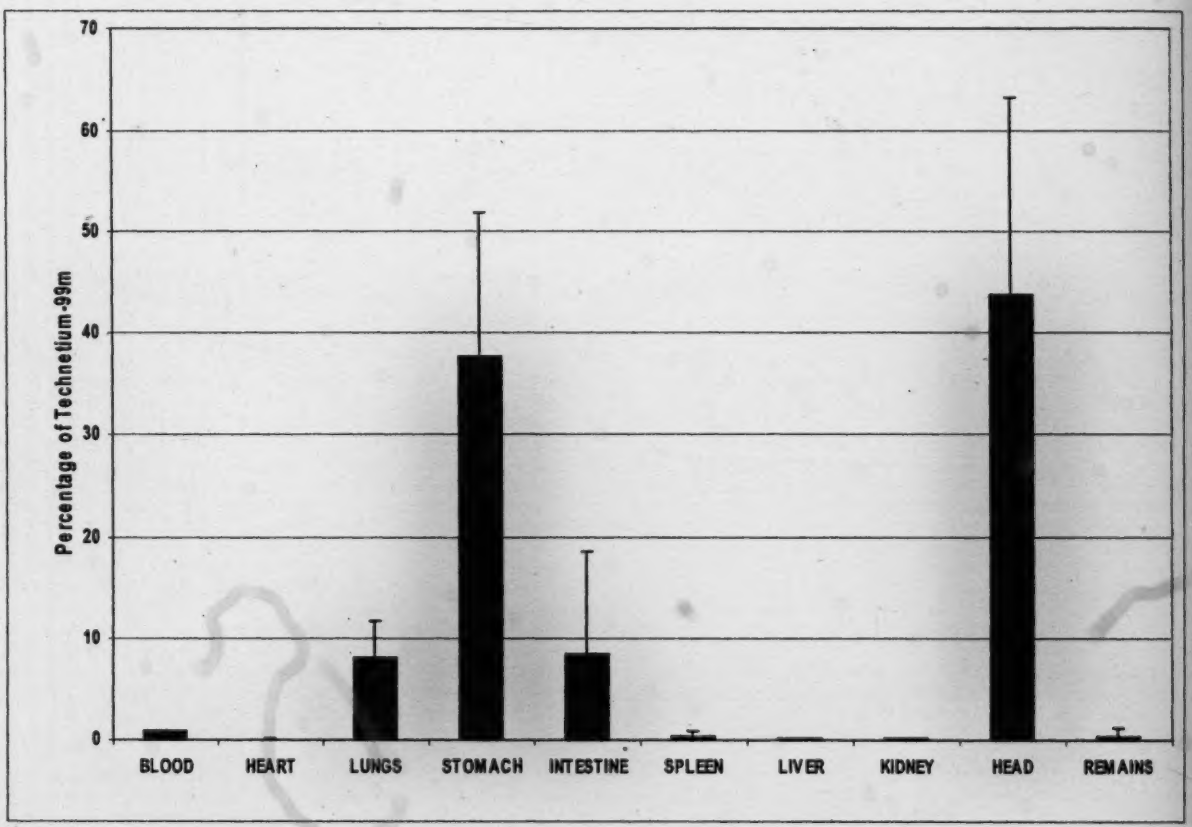

Figure 9: 99mTc labelled HSA deposition (%) in different tissues of the mice after nose-only inhalation (n = 3 sets of exposures with 11 mice in each exposure, error bars indicate ± SD)[*].

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4.6 Comment on the nose-only inhalation chamber for aerosol delivery

Of the total activity placed in the nebulizer, 0.108 ± 0.027% was deposited in the mice in total during 10 minutes of nebulization, and 0.0087 ± 0.0021% was deposited in the lungs. Based on the results shown in Table 5 for the percentage of aerosol nebulized, assuming a minimum of 40% of aerosol was available in the chamber, no more than 0.27 ± 0.67% of the aerosol delivered to the chamber was deposited in the mice, and 0.0216 ± 0.005 % reached the lungs. For example, if 10 μg of drug were needed in the lungs to achieve the desired therapeutic effect, this setup would need 100 mg of the drug for the nebulization process. This method may still be a promising approach for delivery inspite of its limitation in terms of the drug needed. This chamber can be considered a significant delivery device in situations where the drug concentrations in the lungs cannot be achieved by other routes of administration or other modes of aerosol delivery, for some of the drugs administered by other routes may not be systemically stable and may result in degradation of the substance before it can act on the lungs, which are the target organs.

These results also showed that delivery of aerosols by this nose-only inhalation chamber was extremely inefficient in terms of the amount of drug that reached the mice's lungs and bodies. For example, if a drug has only a biological source of production and if the sources can produce only a few micrograms of the drug, using that drug for nose-only inhalation chamber studies is not feasible. Moreover, if this drug also has a high index for achieving the minimum therapeutically effective concentration, large quantities of drug may be required for adequate drug levels. This also means the need for longer nebulization times for higher deposition of the drug using this set-up. Even though longer

nebulization times are possible this may cause heavy stress to the mice inhaling aerosols in the chamber.

A nose-only inhalation chamber may not be a feasible way to deliver aerosol if the drug is expensive or if the drug is needed in high doses to attain minimum effective therapeutic concentrations for therapeutic response. The deposition to some extent can increase by changing the formulations characteristics and experimental conditions but it is highly improbable to achieve 100% depositions only in the lung without extra thoracic depositions with this chamber. It has been shown that the deposition fraction in the lung increases by mixing carbon dioxide (5%) into the inhaled air. This may be due to the changed breathing pattern of the mice (Koshkina et al 2001). However, if other than normal air is used for breathing, issues related to toxicity to lung tissues or induced higher stress states in animals or human patients would have to be addressed.

4.7 Mathematical simulation

Regional deposition fractions vs. particle sizes are shown in Figure 10. These results were compared with the work of Phalen (Phalen 1991) and Hsieh et al (Hsieh et al 1999). The results showed that the current computer program prediction for the tracheobronchial deposition fraction compared well with that of Phalen (Phalen 1991) and that the alveolar deposition gave slightly higher values than those given by Hsieh et al. (Hsieh et al 1999) for particle sizes between 0.1 to 1.0 μm. For particle sizes greater than 2.0 μm, the deposition fraction for the alveolar region was lower than that presented by Hsieh et al (Hsieh et al 1999). Furthermore, differences also occured for tracheobronchial deposition. These differences may have resuted either because Hsieh et

al. (Hsieh et al 1999) did not use the last generation of Phalen's (Phalen 1991) mouse lung morphometric model, or because these researchers used different deposition relations for the various deposition mechanisms in the pulmonary region of the lungs than were used in this present study. Table 7 shows the calculated dependence of the deposition fraction on breathing parameters and demonstrates that the deposition increased when the breath frequency increased. Also shown is the variation in deposition for a range of individual particle size distributions. The last four rows of this table use the average values of the MMAD and GSD measured in the inhalation chamber.

4.8 Comparison of experimental deposition with mathematical simulations

The result obtained for lung deposition from the actual experimental tests performed in this study was 8.19 ± 3.56% of the total dose inhaled, which compared favorably with the value from our simulation shown in Table 7. Statistical analysis showed that no significant difference occurred between the experimental aerosol deposition and the mathematical simulation data based on the average measured MMAD and GSD (i.e., the last 4 rows of Table 7). The variation in the experimentally determined data can be accounted for by the various factors on which aerosol deposition depends, i.e., lung physiology, morphology, breathing (Sweeney & Brain 1991; Smaldone 2000) and aerosol size. In spite of this variability, the computer simulation data showed reasonable agreement with the experimental deposition data.

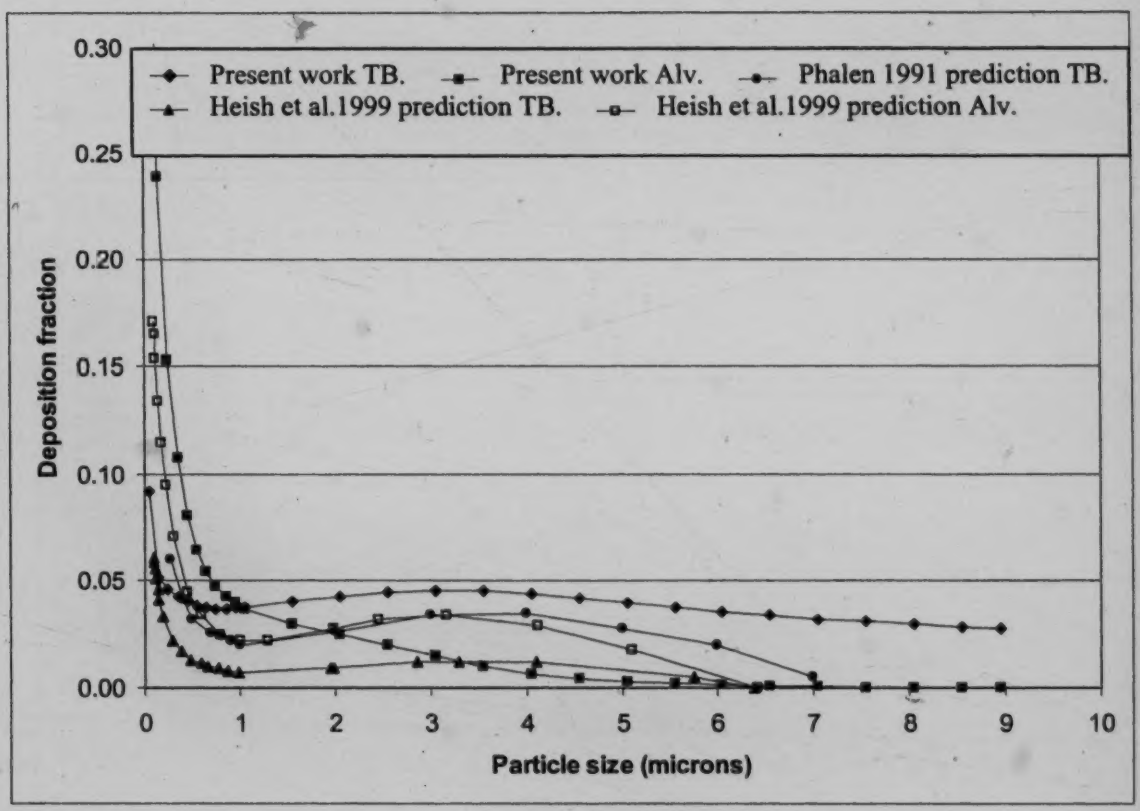

Figure 10: Regional deposition fraction of drug in the respiratory tract of a mouse from mathematical simulation. TB. is tracheobronchial deposition and Alv. is alveolar deposition*.

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Table 7: Deposition fraction as a percentage of total drugs deposited in the respiratory tract of a mouse for various aerosol characteristics and breathing parameters. The last 4 rows are for the average measured MMAD and GSD*.

(* Literature Values)

Deposition Fraction	Aerosol Characteristics	Breathing Parameters*		
		Breathing frequency	Tidal Volume	Inspiratory flow
%	MMAD GSD		mL	mL/s
4.4	4.6 1.57	300	0.289	2.89
4.45	4.6 2.0	300	0.289	2.89
4.73	4.0 2.0	300	0.15	1.5
5.45	4.0 2.0	160	0.289	1.54
6.02	4.0 2.0	300	0.289	2.89
6.13	2.0 1.57	300	0.289	2.89
6.31	3.7 2.0	300	0.289	2.89
7.06	2.31 1.57	160	0.289	1.54
7.69	2.31 2.28	160	0.289	1.54
5.89	3.9 1.43	300	0.289	2.89
5.53	3.9 1.43	160	0.289	1.54
4.85	3.9 1.43	300	0.15	1.5
4.40	3.9 1.43	160	0.15	0.8

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CHAPTER 5: CONCLUSIONS

The study demonstrated that radiolabelled Human Serum Albumin formulation was stable during the process of nebulization. The measurable amounts of drug deposited in the lungs of the mice by using the present nose-only inhalation chamber indicated that it may be successfully used to deliver therapeutic biological molecules. However, only a small fraction of the drug was deposited in the lungs of the mice (0.01% of the drug in the nebulizer), whereas most of the inhaled drug was deposited in the extrathoracic space, with considerable delivery to the stomach and upper GI tract during the period of the study. This result would be a concern in situations like vaccine delivery where gastrointestinal deposition is an issue. Our data suggests that the present nose-only device may not be the first choice for delivery devices because of the large amounts of drug required for nebulization. Indeed, in order to deliver expensive drug molecules with this chamber, it would need further improvement to increase the deposition in the lungs. Although mice are difficult laboratory animals to use to study aerosol delivery, they provide important models for the preliminary efficacy screening of drug molecules and pharmaceutical delivery systems for a number of diseases. Ignoring mice as an animal model for aerosol delivery would be unwise, since in some instances they provide the most appropriate disease model for the preclinical evaluation of a drug. The present mathematical simulation for aerosol deposition gave a good prediction of the lung deposition of the formulation. Further, the mathematical simulation will help in predicting the aerosol deposition of drug candidates in the lungs of mice, thereby minimizing the required number of *in vivo* animal experiments.

A version of this chapter has been published. Nadithe et al.2003. Journal of Pharmaceutical Sciences. 92: 1066–1076.

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